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The phenylpropenamide derivative AT-130 blocks HBV replication at the level of viral RNA packaging

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Abstract

Nucleos(t)ide analogue antiviral therapy for chronic hepatitis B has proven to be effective in the short term but the frequent development of resistance limits its clinical utility. Agents targeting other stages of viral replication are needed in order to develop improved combination therapies. The phenylpropenamide derivatives AT-61 and AT-130 have been shown to inhibit HBV replication *in vitro,* but the mechanism of action of these compounds remains undefined. The aim of this study was to determine the mechanism of action of AT-130, a non-nucleoside inhibitor of HBV in several *in vitro* models of replication. These studies found that AT-130 inhibited HBV DNA replication in hepatoma cells but had no effect on viral DNA polymerase activity or core protein translation. Total HBV RNA production was also unaffected in the presence of the drug whilst the amount of encapsidated RNA was significantly reduced, thereby inhibiting subsequent viral reverse transcription. These studies have established that the inhibition of HBV genome replication by a non-nucleoside analogue acting at the level of viral encapsidation and packaging is a potentially useful strategy for future therapeutic drug development in the management of chronic hepatitis B.

Keywords: Hepatitis B virus; Drug resistance; Phenylpropenamide; Viral RNA packaging

1. Introduction

Despite the presence of an effective vaccine, hepatitis B virus (HBV) infection remains a global public health problem with over 400 million people chronically infected worldwide (Lee, 1997). These individuals are at risk for developing progressive liver disease, cirrhosis and hepatocellular carcinoma which leads to approximately 1 million deaths annually (Beasley, 1988; Lee, 1997).

Although various treatment options exist for chronic HBV infection, none is entirely satisfactory. Interferon (IFN) alpha has both immunostimulatory and direct antiviral properties, but is effective in only one-third of patients (Wong et al., 1993); and treatment is greatly hampered by significant adverse effects (Lok and McMahon, 2004). The use of longer acting pegylated interferon has resulted in an incremental increase in efficacy (Lau et al., 2005). Important advances have been made with the introduction of non-toxic, efficacious nucleos(t)ide analogues that interfere directly with HBV genomic DNA synthesis. Lamivudine (LMV), adefovir (ADV), and entecavir (ETV) are well-tolerated and lead to viral suppression in the majority of treated patients while therapy is maintained (Lai et al., 1998; Dienstag et al., 1999; Hadziyannis et al., 2003; Marcellin et al., 2003; Lok and McMahon, 2004).

Unfortunately the clinical utility of these agents, particularly LMV, is limited by the frequent development of resistance, occurring at rates of up to 15–30% per year (Liaw, 2001; Lai et al., 2003), such that by four years of therapy over 70% of treated patients show evidence of LMV resistance. For ADV, the emergence of resistance is slower, however 28% of treated patients develop genotypic resistance by year 5 (Hadziyannis et al., 2005). To date, ETV resistance has been described in the setting of previous LMV resistance (Tenney et al., 2004) occurring at approximately 10% per annum (Gish, 2005), and a much lower level of 1% in naïve patients by year 3 (Colonno et al., 2006).

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The problem of antiviral drug resistance is not unique to HBV. Control of resistance in human immunodeficiency virus (HIV) treatment has been achieved by using combination therapy with agents targeting different stages in the viral lifecycle (Shaw and Locarnini, 1999; Malik and Lee, 2000). Likewise, polymerase-independent inhibitors of HBV replication such as nucleocapsid-binding agents, will be important additions to the current HBV armamentarium (Deres et al., 2003). In 1998 King et al. described AT-61, a phenylpropenamide derivative, with specific anti-HBV activity that appeared to interfere with the encapsidation process. A related compound, AT-130, was found to be a more potent inhibitor of HBV replication and both agents have also been shown to be effective against LMV-resistant HBV mutants (Delaney et al., 2002). However, the mechanism of action of AT-130 has remained unclear. We report here that AT-130, like AT-61, appears to act by inhibiting the packaging step in the lifecycle of HBV.

2. Materials and methods

2.1. Compounds and HBV clones

AT-130 ((E)-*N*-(3-bromo-3-(2-methoxyphenyl)-1-oxo-1-(piperidin-1-yl)prop-2-en-2-yl)-4-nitrobenzamide) and AT-61 ((E)-*N*-(1-chloro-3-oxo-1-phenyl-3-(piperidin-1-yl)prop-1-en-2-yl)benzamide) was synthesized as previously described (Perni et al., 2000) and supplied by Gilead Sciences (Foster City, CA) (see Fig. 1A). Both compounds were prepared in DMSO as a 5 mM stock solution and kept at 4 °C. LMV was also provided by Gilead Sciences. A stock solution of 50 mM LMV was prepared in sterile water and kept at 4 °C. Dilutions of the drugs were performed on the day of medium change. Genotype D 1.3 unit-length HBV (wild-type) was kindly provided in pBluescript II SK (Stratagene, La Jolla, CA) by Professor H. Schaller (University of Heidelberg, Heidelberg, Germany). This vector was subcloned into pBlueBac 4.5 and used in the recombinant HBV-baculovirus system as initially described by Delaney and Isom (1998) and recently modified (Chen et al., 2003).

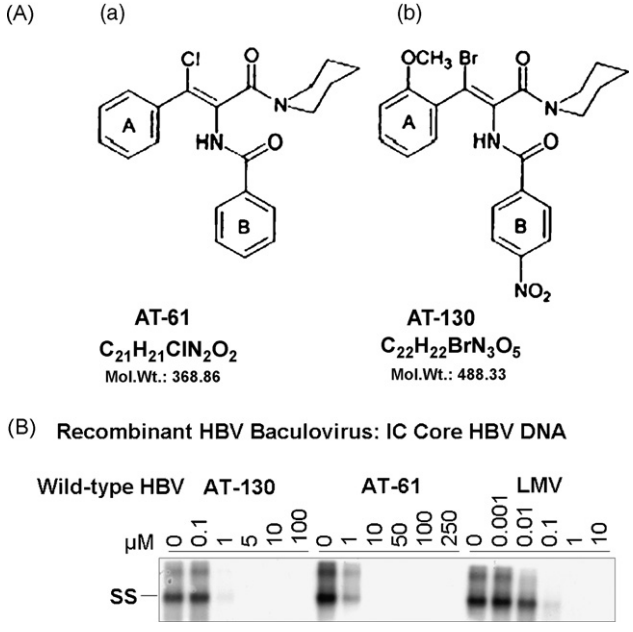

Fig. 1. (A) Chemical structures of AT-61 (a) and AT-130 (b). Mol. Wt. = molecular weight (Modified from Delaney et al., 2002). (B) Southern blot of core associated HBV DNA from cell cultures transduced with recombinant HBV baculovirus and treated with increasing doses of AT-61, AT-130 and LMV (SS = single stranded).

2.2. Cell culture and transient transfection

The HepG2 cell line was maintained at 37 °C in humidified incubators in 5% CO_2 and cells were fed minimal essential medium (MEM Invitrogen, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (FBS). The Huh-7 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM – Invitrogen, Carlsbad, CA) supplemented with 10% FBS, and incubated at 37 °C in humidified incubators in 5% CO_2. The pTRE-Huh-7 cell lines expressing the HBV precore or core protein or control are described in detail below. The Huh-7 and pTRE-Huh-7 cell lines were transfected using FuGene 6 reagent (Roche Molecular Biochemicals, Mannheim, Germany) following the Manufacturer's recommendations and as described previously (Chin et al., 2001).

2.3. HBV baculovirus production and infection of HepG2 cells

Replication-competent genotype D 1.3 unit length recombinant HBV baculovirus, described above, was generated and purified as previously described (Delaney and Isom, 1998). HepG2 cells were seeded to semiconfluence in 60 mm diameter tissue culture dishes and allowed to adhere overnight. The following day, an average cell count was calculated from 3 dishes and used to determine the volume of high-titer viral stock necessary to infect the cells at a multiplicity of infection (moi) of 50 (Delaney and Isom, 1998). Recombinant HBV-baculovirus was diluted in serum-free MEM and adsorbed to the HepG2 cells for 1 h at 37 °C with gentle rocking every 15 min to ensure even distribution. The inoculum was then aspirated and HepG2 cells were washed twice with phosphate-buffered saline (PBS) and refed MEM-FBS containing the required drug concentrations. Media was replaced every two days with drug-containing media until harvest at day seven.

2.4. Analysis of replicative intermediates

2.4.1. HBV DNA and RNA containing core particles

HBV core particles were isolated from HepG2 cells as described previously (Delaney and Isom, 1998; Delaney et al., 2002). Briefly, after removing the supernatant, cells were washed with PBS and then lysed with 800 μl of 0.5% Nonidet P-40 in PBS for 20 min at 4 °C. Cell lysates were then transferred to microfuge tubes and spun at 13,000 rpm for 5 min to remove the nuclei. Unprotected DNA was removed by incubating the samples with 20 U of RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) for 1 h at 37 °C. The incubation mix-

ture was then adjusted to 10 mM EDTA, 1% SDS, and 100 mM NaCl. Proteinase K was then added to the samples at a final concentration of 0.5 mg/ml and incubated for a further 4 h at 37 °C. Replicative intermediates were then isolated following sequential phenol/chloroform extractions and isopropanol precipitation as described previously (Delaney and Isom, 1998; Delaney et al., 2002). Precipitated nucleic acids were resuspended in 5 mM EDTA and digested with 20 U of DNase-free RNase I (Roche Diagnostics) for 1 h at 37 °C (Sambrook and Fritsch, 1989). Replicative intermediates were then analysed by electrophoresis, Southern blotting and autoradiography as described previously (Delaney and Isom, 1998; Delaney et al., 2002) using strand-specific riboprobes for HBV plus-strand and minus-strand DNA (Blum et al., 1984).

2.4.2. Analysis of HBV RNA

Total RNA was isolated from HepG2 cells by guanidine thiocyanate cell lysis using the Qiagen RNA extraction kit, according to the Manufacturer's instructions (Qiagen, Germany). Encapsidated RNA was isolated similarly from HBV cores, following isolation of viral nucleocapsids as described above. The RNA was then reverse transcribed (RT) to make cDNA using Superscript II (Stratagene, La Jolla, CA) and Random Primers following the manufacturer's instructions. The cDNA was then quantified using the LightCycler Real-Time PCR (Roche, Idaho Technology, Idaho Falls). The PCR reaction was performed using primers 5′ CTC CGG AAC ATT GTT CAC CT 3′ (nt 631–650) and 5′ GTT GAT AAG ATA GGG GCA TTT GGT GG 3′ (nt 900–925). (Numbering according to HPBADR1CG; accession number M38454 (Delaney and Isom, 1998)). A parallel reaction was run using the LightCycler β globin control kit (Roche Diagnostics, Mannheim, Germany) as per the Manufacturer's instructions and described in detail previously (Werle-Lapostolle et al., 2004). Results from the HBV data were then corrected for cellular input based on the β globin results. The PCR reaction was performed in a total volume of 20 μl containing 5 μl of cDNA template, 2 μl of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics), 5 mM $MgCl_2$ and 10 μM of each primer. Thermal cycling consisted of an initial denaturation of 95 °C for 10 min, followed by 40 cycles of 95 °C/15 s, 58 °C/10 s, 72 °C/20 s and 81 °C/3 s with acquisition of fluorescence signal during this step. After amplification, a melting curve was generated by holding the reaction at 95 °C/30 s, lowering the temperature to 65 °C/15 s, then increasing to 95 °C at 0.1 °C/s with continuous collection of fluorescence. Melting curves and quantitative analysis of the data were performed using LightCycler analysis software 3.5 (Roche Diagnostics). A standard curve was included in each run using HBV plasmid of known concentration as template.

2.4.3. Analysis of extracellular HBV DNA

Supernatant from HepG2 cells was collected and centrifuged at 3000 rpm for 5 min at 4 °C to remove cellular debris. HBV particles were precipitated by adding 2.5 ml of 26% polyethylene glycol 8000 and incubated overnight at 4 °C (Delaney and Isom, 1998). The following day, precipitation was completed by centrifuging the samples at 14,000 rpm for 20 min at 4 °C. The supernatant was aspirated and the pellets were resuspended in a buffer containing 5.5 mM $MgCl_2$ in 10 mM Tris–HCl (pH 7.5). The samples were then digested with 20 U of RNase-free DNase I (Roche Diagnostics) for 1 h at 37 °C. The reaction was stopped by the addition of a buffer containing 2.5% SDS, 100 mM Tris–HCl (pH 7.5) and 125 mM EDTA. Proteinase K was added to each sample at a final concentration of 0.5 mg/ml and incubated for a further 4 h at 37 °C. DNA was then extracted following sequential phenol/chloroform extractions and isopropanol precipitation as described above. Nucleic acid pellets were then resuspended in 5 mM EDTA and analysis was carried out by electrophoresis, Southern blotting and autoradiography as described above.

2.5. HBV Core and precore protein expression vector cell lines

Huh-7 cells with inducible expression of the HBV core and precore proteins were produced using the Tet-Off™ Gene Expression System (pTRE-2, Clontech, Palo Alto, CA) and were described in detail recently (Locarnini et al., 2005). A third pTRE-Huh-7 cell line without any cloned viral product was used as the parent control cell line (Locarnini et al., 2005). The cells were maintained in DMEM supplemented with 10% FBS, G418 (500 μg/ml) and tetracycline (2 μg/ml) to repress protein expression by the tetracycline response expression system.

2.6. Analysis of secreted HBeAg

Medium from HepG2 cells was collected, centrifuged at 3000 rpm to remove cellular debris and transferred to clean tubes. The amount of HBeAg secreted into the cell culture medium was then determined using a commercially available enzyme immunoassay (Abbott Laboratories, IMX, Chicago, IL).

2.7. Analysis of core antigen and nucleocapsid production

Core antigen was detected from cells by immunoblot using anti-core antibodies purchased from Abcam (mouse-monoclonal) and Dako (rabbit-polyclonal), followed by an anti rabbit or anti-mouse HRP secondary antibody. Protein bands were visualised using chemiluminescence. The cell systems used were the recombinant HepG2 baculovirus and pTRE-Huh-7 precore/core cell lines, as outlined above (Locarnini et al., 2005).

2.7.1. Nucleocapsid detection

Nucleocapsids were isolated and purified over a sucrose density gradient and visualized by immunoelectronmicroscopy (see below). For the Western blot analysis of nucleocapsids, pTRE Huh-7 cells expressing the core protein (see above) were subcultured into 60 mm dishes. Each dish was treated 24 h later with either TNF-alpha (100 ng/ml) or AT-130 (IC_{50} and IC_{90}, as determined in earlier experiments) with and without tetracycline for each treatment sample (as described above) for a

further 7 days. Core particles were isolated following lysis in a buffer containing 0.5% Nonidet P-40 in PBS and the nuclei were removed by centrifugation at 13000 rpm. The cell lysates were then stored at 4 °C. Lysates were subjected to electrophoresis on a non-denaturing 12% polyacrylamide gel in Tris-glycine HCl pH 8.0 with a 6% stacking gel in the same buffer followed by Western blot analysis using polyclonal anti-HBc (see above) as per standard procedures.

2.7.2. Core protein detection

Cells were harvested after five to seven days using a 2% SDS lysis buffer containing complete protein inhibitors and mixed with 5× Laemmli loading dye as previously described (Delaney and Isom, 1998; Delaney et al., 2002; Chen et al., 2003). Proteins were run on a 12% polyacrylamide gel, transferred to Hybond-C membrane and detected using either monoclonal or polyclonal anti-HBc.

2.8. Packaging assay for HBV pregenomic RNA

pTRE Huh-7 cells expressing the core or precore protein or control (Locarnini et al., 2005) were grown in 100 mm dishes. Each cell line was then transfected with FuGene 6 reagent with wildtype 1.3 genomes length HBV of genotype D plasmid, and a core stop codon construct created by converting the sixth amino acid in the core coding sequence to a stop codon. The core stop codon mutant is unable to replicate *in vitro* without the provision (rescue) of HBV core protein *in trans*. The plasmid pBlueBac 4.5 with no modification or insertion was used as the positive control. To study the packaging reaction in these cell lines, half the cells were exposed to 2 μg/ml of tetracycline, whilst the other half remained tetracycline-free in order to allow induction of precore, core or mock protein expression. At least 2 h prior to transfection, cells were exposed to the IC_{50} concentration for HBV of AT-130 (see Fig. 1B), LMV or no drug. Each of the three cell lines was then transiently transfected with the DNA constructs using FuGene 6 (Roche Diagnostics) as described previously (Chin et al., 2001). Cells were maintained in media containing the required drug concentrations with or without tetracycline and replaced every two days until harvest on day five post-transfection.

Core particles were isolated from the cells following lysis in a buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 0.5% Nonidet P-40 and the nuclei pellet removed. The cell lysates were then treated with 20 U RNase-free DNase I (Roche Diagnostics) and 20 U DNase-free RNase I (Roche Diagnostics) for 1 h at 37 °C. This step was repeated to ensure complete digestion of input DNA. Further digestion was stopped with the addition of a one fifth volume of a buffer containing 2.5% SDS, 100 mM Tris–HCl (pH 7.5) and 125 mM EDTA. The sample was then either used for extraction of HBV DNA or HBV RNA as follows: HBV DNA was extracted using phenol:chloroform following proteinase K digestion, and then analysed by electrophoresis, Southern blot hybridization and autoradiography as described above. HBV RNA was extracted from the core sample using a commercial guanidine isothiocyanate method, RNAqueous™-4PCR, following the Manufacturer's instructions (Ambion, Texas). The resulting nucleic acid was divided and one aliquot was subjected to DNase I (Ambion, Texas) treatment before reverse transcription PCR to construct cDNA and then analysis by quantitative PCR using the LightCycler as described above. The other aliquot was tested directly for HBV DNA by real time-PCR also using the LightCycler.

2.9. Endogenous HBV DNA polymerase assay

Isolated, partly purified intracellular HBV core particles as well as mature virions were used as the source of HBV DNA polymerase activity. Extracellular virions and intracellular core particles were generated from cell cultures of Huh-7 or HepG2 cells following transfection of the cells with a recombinant plasmid containing a full length copy of the HBV genome. Following harvest, virions and core particles were treated with 20 U RNase-free DNase I (Roche Diagnostics) and precipitated with PEG 8000 as previously described (Delaney and Isom, 1998). The particles were resuspended in a polymerase buffer containing 50 mM Tris–HCl (pH 7.5), 75 mM NH_4Cl, 1 mM EDTA, 20 mM $MgCl_2$, 0.1 mM β-mercaptoethanol, 0.2% (v:v) Nonidet P-40. The enzyme stock was stored in small aliquots at −70 °C.

The polymerase assay mixture contained 45 μl enzyme preparation in polymerase buffer and 5 μl of drug concentration or nuclease-free water. Foscarnet, a non-nucleotide RT inhibitor (final concentration 50 μM), was included in each set of assays as the positive control. Varying concentrations of AT-130 or foscarnet were added to the mixture. Polymerase reactions were initiated by the addition of 1 μl [α-^{32}P]-dNTP mixture. After 4 h incubation at 37 °C, reactions were stopped by addition of 5 μl 10% (v:w) SDS and proteinase K solution to a final concentration of 2.5 mg/ml and incubated for a further 2 h at 37 °C. HBV DNA was then extracted with sequential phenol:chloroform, precipitated with isopropanol, redissolved and electrophoresed through 1% TAE agarose gels. Gels were dried and autoradiographed at −70 °C with the aid of intensifying screens. Three separate sets of experiments were performed. The final concentration of each [α-^{32}P]-dNTP in the reaction mixture was approximately 0.0017 μM. AT-130 was tested at five different (final) concentrations: 0.005, 0.05, 0.5, 5 and 50 μM.

2.10. Immune electron microscopy

Aliquots of core preparations from the hepatoma cell lines described above were processed for immune electron microscopy (IEM) using anti-HBc antibody (Dako polyclonal) with the methodology described by Locarnini et al. (1974). Grids of 400 copper mesh were carbon-coated and a drop of the IEM pellet was then added. Grids were washed with PBS before drying and staining with 3% (w/v) phosphotungstic acid pH 7.4. Grids were examined in a Phillips EM301 microscope at a plate magnification of 40,000 times. Electron microscopy and IEM are not quantitative assays and only permit general trends in terms of data interpretation.

3. Results

3.1. AT-130 inhibits HBV DNA synthesis

To determine the antiviral activity of AT-130 and AT-61, recombinant HBV-baculovirus at a moi of 50 pfu/cell, was transduced into HepG2 human hepatoma cells with increasing concentrations of AT-130, AT-61 and LMV. At day 7 the cells were harvested and the replicative intermediates were measured. As shown in Fig. 1B and similar to previously published results (Delaney et al., 2002), AT-130 at a concentration of 2.5 μM, reduced encapsidated HBV DNA by 50% (IC_{50}) and at 18.5 μM by 90% (IC_{90}). AT-130 was more active than AT-61 but not as active, on a molar basis, as LMV. All three agents also effectively inhibited production of mature extracellular virions (data not shown). AT-130 was not toxic in HepG2 or Huh-7 cell lines at doses up to 250 μm up to 7 days (data not shown).

3.2. AT-130 does not significantly affect HBV DNA polymerase activity

LMV, ADV, ETV and other nucleoside/nucleotide analogues inhibit HBV DNA synthesis by interfering directly with the reverse transcription process. To test if this was the mechanism of action of AT-130, the endogenous HBV DNA polymerase activity assay was performed. Mature excreted virions and intracellular core particles were isolated and then incubated in the presence or absence of varying concentrations of AT-130 or foscarnet as positive control. As seen in Fig. 2, even with the addition of high concentrations of AT-130, only a slight reduction in HBV DNA synthesis by intracellular core particles was observed, indicating that AT-130 does not inhibit HBV DNA synthesis by blocking the HBV endogenous DNA polymerase reaction directly. Foscarnet, a known polymerase inhibitor was used as a positive control and showed marked inhibition of HBV DNA synthesis.

3.3. AT-130 has no effect on total HBV RNA production but does reduce encapsidated RNA

To assess whether AT-130 had an effect on the viral RNA transcription in recombinant HBV baculovirus transduced HepG2

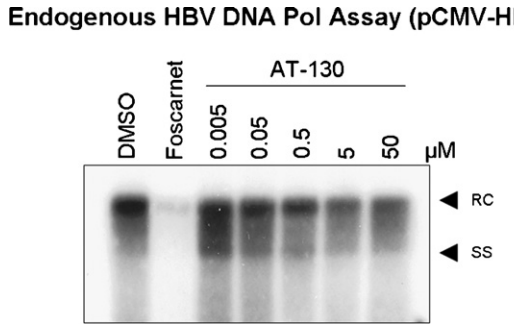

Fig. 2. Endogenous HBV DNA polymerase assay from transiently transfected Huh 7 or HepG2 cells with the plasmid pCMV-HBV in the presence of increasing concentrations of AT-130 and a single dose of Foscarnet (50 μM). The results shown are from intracellular core particle preparations (rc = relaxed circular, ss = single stranded).

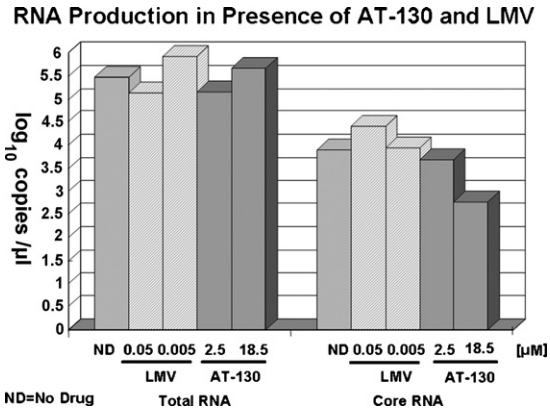

Fig. 3. Graphic representation of RT and qPCR data of total and core RNA from HepG2 cells transduced with wt recombinant HBV baculovirus exposed to AT-130 (IC_{50} and IC_{90}) and LMV (IC_{50} and IC_{90}).

cells, total RNA production was measured using quantitative real-time reverse-transcription PCR (RT PCR). Data was corrected for cellular input also by measuring β globin cDNA. As shown in Fig. 3, AT-130 had no effect on total RNA production implying that it has no effect on RNA transcription. LMV similarly did not affect total RNA production. Prior to HBV DNA synthesis, the pre-genomic RNA must first be packaged within the nucleocapsid. To assess whether the production of encapsidated RNA was reduced, core particles were isolated from infected cells, the RNA extracted, and then quantified using real-time RT PCR. As shown in Fig. 3, increasing concentrations of AT-130 from the IC_{50} to the IC_{90} level showed a dose-dependent inhibition of the production of encapsidated RNA. In contrast, LMV had no effect on packaged RNA levels. The reduction in encapsidated but not total HBV RNA, indicates that AT-130 might be acting at the level of pregenomic RNA packaging into nucleocapsids. These findings were confirmed in a repeat experiment.

3.4. AT-130 does not affect core protein or nucleocapsid production

To ensure that the above observation of reduced encapsidated RNA was not the result of an inhibitory effect on core protein translation or nucleocapsid assembly, core protein production was measured by immunoblot hybridisation in HepG2 cells transduced with recombinant HBV baculovirus at a moi of 50. As shown in Fig. 4, even at the IC_{90} concentration (18.5 μM), AT-130 had minimal effects on core protein production. At very high doses, LMV exposure did reduce core protein translation, an effect of nucleoside analogue therapy which has been observed previously (Luscombe et al., 1996).

3.5. AT-130 does not affect the activity of the protein expression vector

To further explore the possibility that AT-130 affected the encapsidation process, the pTRE-Huh-7 core and precore expression cell lines as well as control cells were exposed to increasing concentrations of AT-130 or LMV in the presence

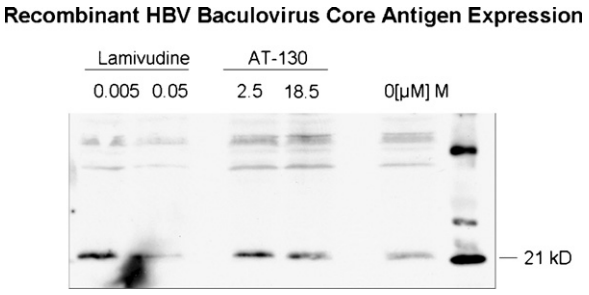

Fig. 4. Immunoblot of core protein (21 kDa band) from HepG2 cells transduced with wt recombinant HBV Baculovirus exposed to LMV (IC_{50} and IC_{90}) and AT-130 (IC_{50} and IC_{90}).

(TET-on inhibition) or absence (TET-off synthesis) of tetracycline. As seen in Fig. 5A, neither AT-130 nor LMV had any effect on the synthesis of the 21 kDa core protein following induction. Almost identical results were found for the pTRE-Huh-7 precore expressing cell line and HBeAg production (data not shown).

In order to examine an effect on nucleocapsid assembly from newly translated core protein, pTRE Huh-7 cells expressing the core protein were treated with either TNF-alpha or AT-130 with (+) or without (−) tetracycline treatment for 7 days. Core particle preparations were then made using 0.5% NP-40 as described in Section 2 and this material, which contains both nucleocapsids and newly translated core protein, was analysed on a non-denaturing 6% stacking (nucleocapsids) and 12% separating (core protein) (see Fig. 5B) as detailed in Section 2. TNF-α treatment is known to slow HBV replication by reducing core nucleocapsid assembly (Biermer et al., 2003). Comparison of lanes 1 with 3 and 2 with 4 shows that TNF-α treatment reduced the nucleocapsid band, whilst treatment with AT-130 at either IC_{50} (lane 5) or IC_{90} (lane 6) resulted in no change (Fig. 5B).

Finally, similar amounts of 27 nm core particles were visualised in either LMV or AT-130 treated cultures of the pTRE-Huh-7 core cell line using immunoelectron microscopy, indicating that neither treatment substantially affected nucleocapsid formation (Fig. 6).

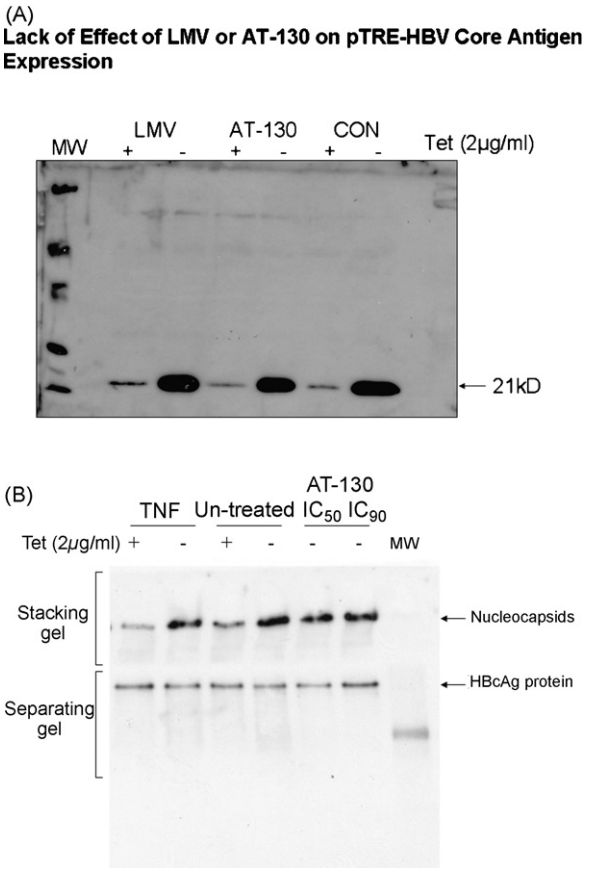

Fig. 5. (A) Immunoblot of pTRE-HBV Core (21 kDa band) antigen expression from cells exposed to LMV (IC_{90}) and AT-130 (IC_{90}). Core protein production was not altered by AT-130 or LMV treatment. (B) Non-denaturing immunoblot of core protein expression from pTRE-HBV core cell line after exposure to TNF (100 ng/ml) and AT-130 (IC_{50} and IC_{90}). The analysis of the NP-40 core preparations was performed onto 6% acrylamide stacking gel (nucleocapsids) and 12% separating gel (core protein) in Tris-glycine HCl pH 8.0 as described in Section 2.

3.6. *Rescue of the replication of the core stop codon mutant by the pTRE core expression vector cell line is inhibited by AT-130*

A greater than full length cDNA HBV clone with a stop codon inserted in the core gene was transfected into the core expression cell line in order to measure the ability of the cell line to provide functional core protein *in trans*. This cDNA mutant is normally replication incompetent due to its inability to produce core protein. The core-stop codon mutant or the wild-type virus replication competent plasmid were individually transfected into each of the three expression vector pTRE Huh-

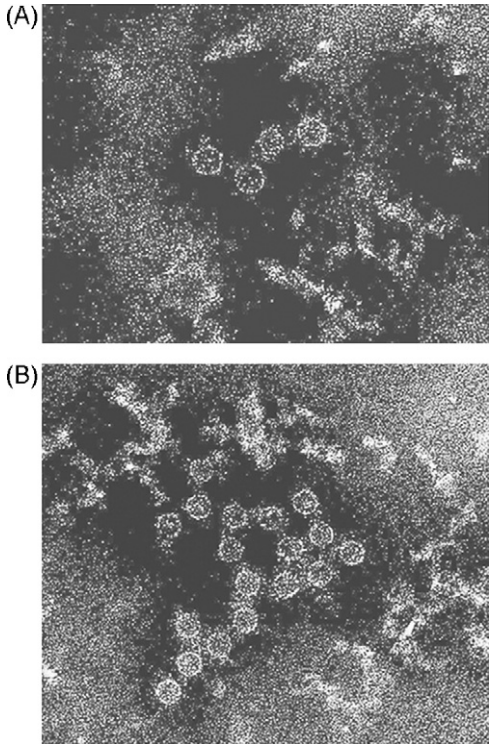

Fig. 6. Immune electron micrographs of core particles from cultures treated with no AT-130 (A) or treated with AT-130 (B) for five days. Twenty-seven nanometer (nm) particles of the HBcAg were readily visualized in both preparations.

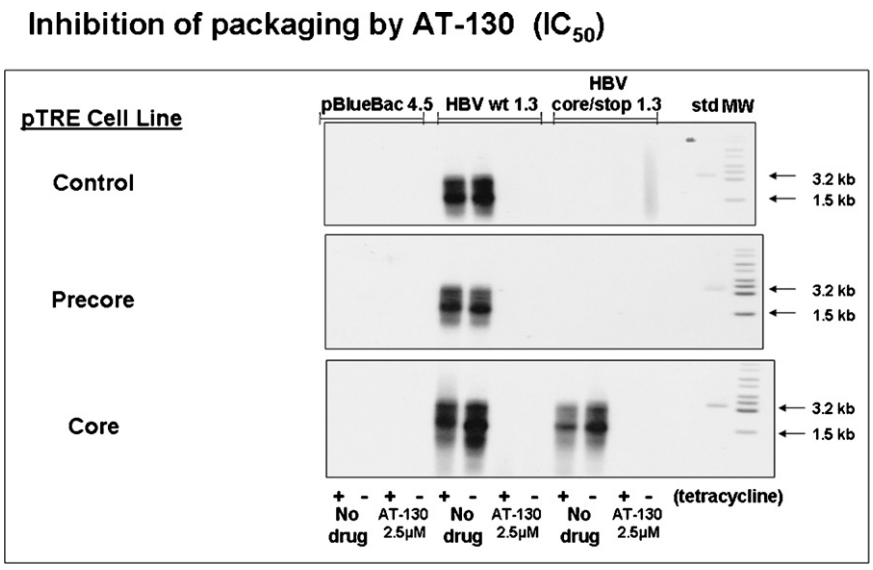

Fig. 7. Inhibition of packaging by AT-130. Southern blot of IC HBV DNA production from the three pTRE-HBV cell lines transiently transfected with the plasmids pBlueBac 4.5, HBV wt 1.3 and core/stop 1.3. Cells were treated with AT-130 (2.5 μM) or no drug.

7 cell lines (core, precore, negative control with pTRE alone) and encapsidated HBV DNA was measured by Southern blot (Fig. 7). As expected, wild-type HBV was able to replicate in all three cell lines with or without tetracycline present in the cell culture medium. When the core-stop codon mutant was transfected into the control (pTRE alone) and precore cell lines, no replication occurred, confirming its replication incompetence due to a lack of core protein. When transfected into the core producing cell line in the tet-off mode, productive HBV replication was restored as demonstrated by a marked increase in core-associated HBV DNA synthesis. In the presence of tetracycline, only a small amount of DNA synthesis was detected. This is due to leakiness of the core expression vector. (See Fig. 5 tet + lanes for comparison).

Each of the transfections described above was repeated but performed in the presence and absence of AT-130 and LMV at IC_{50}. Again, AT-130 inhibited HBV DNA synthesis by wild-type HBV and also inhibited replication of the core-stop codon mutant despite the availability of core protein from the expression vector, indicating that AT-130 had inhibited this packaging reaction *in trans*. When exposed to LMV, there was no inhibition of the packaging reaction (data not shown).

3.7. AT-130 blocks encapsidation of pgRNA

To further resolve the site of action of AT-130, the pTRE-core expressing cell line was again transfected as in the experimental design of Section 3 above as outlined in Fig. 7, but this time encapsidated RNA was quantified using the real time RT PCR for HBV RNA. In the presence of AT-130, encapsidated RNA was below the level of detection for both wild-type and core-stop codon mutant HBV in all three cell lines (Fig. 8). In contrast, while LMV decreased core-associated HBV DNA synthesis as expected, it had no effect on encapsidated RNA production. These findings were confirmed in a repeat experiment. These results indicate that AT-130, in contrast to LMV, directly inhibited the encapsidation process of pregenomic RNA into potential core-associated replication complexes.

4. Discussion

The present study confirms the original observation of King et al., 1998 that AT-61 inhibits HBV replication and provides evidence that the related compound AT-130, a phenylpropenamide derivative, appears to inhibit HBV replication by interfering with the encapsidation process. Successive stages of the replication cycle of HBV post-transcription of pgRNA were examined for sensitivity to the effects of AT-130. AT-130 inhibited the synthesis of both intracellular core associated HBV DNA and mature extracellular virus to a similar degree, implying that the compound does not block the egress of the mature virion. The compound had no effect on HBV DNA polymerase activity indicating that it does not behave as a reverse transcriptase inhibitor. Most importantly, production of encapsidated RNA was significantly reduced by AT-130 while no effect on total RNA transcription was seen, indicating that AT-130 affected the packaging of the pgRNA into nucleocapsids. Furthermore, it was shown that AT-130 did not reduce the level of core translation or affect the process of nucleocapsid formation.

Using the pTRE-protein expression vector Huh-7 cell line making HBV nucleocapsids, the encapsidation process was examined more closely. The core protein expression vector was able to rescue replication of a core-deficient HBV mutant *in trans*, and AT-130 blocked this rescue suggesting that the compound interfered directly with the packaging process. This occurred without affecting the level of core antigen production or the formation of the 27 nm nucleocapsids. The precise mechanism by which AT-130 affects encapsidation is not clear. It may cause steric interference or, alternatively, it may interact with the host cell chaperone proteins such as Hsp 90, which stabilize the pgRNA polymerase interaction necessary for encapsidation to proceed (Wang and Seeger, 1992; Seeger and Mason, 2000).

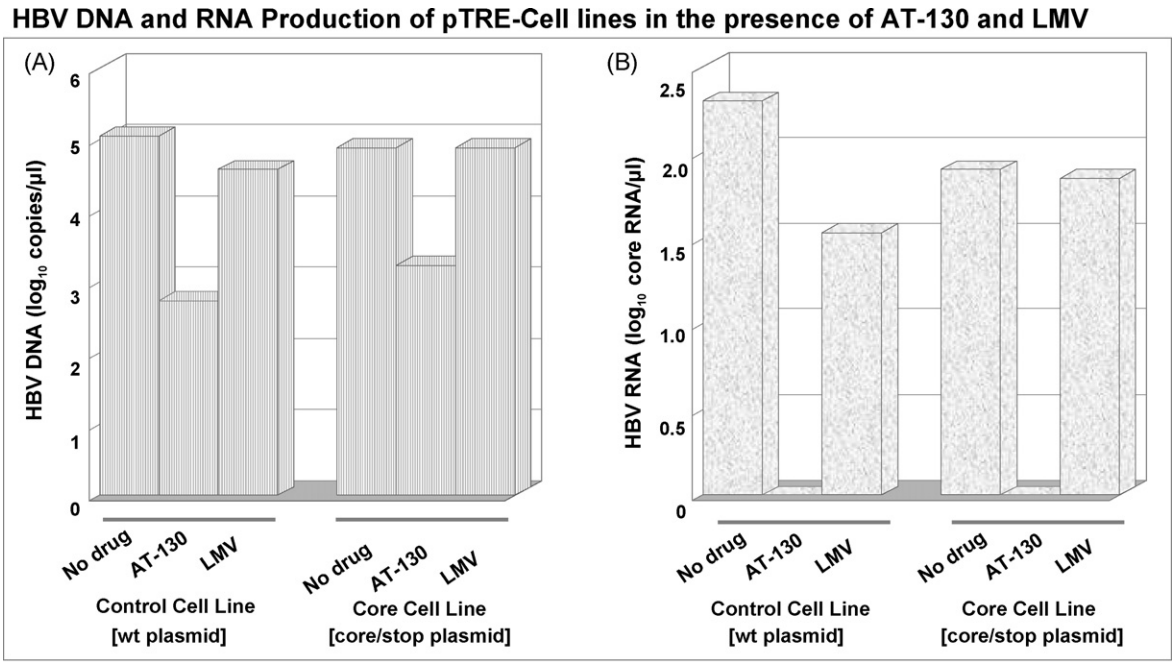

Fig. 8. Graphic representation of HBV DNA (A) and RNA (B) from pTRE-HBV control and core cell lines transiently transfected with the plasmids wt 1.3 and core/stop 1.3. Cells were treated with AT-130 (2.5 μM), LMV (0.1 μM), or no drug.

If steric interference were involved, it might be expected that a greater affect would be seen when core protein is provided *in trans* rather than *in cis*. The core-deficient mutant was provided with core exclusively *in trans* (by the expression vector), while wild-type HBV used core provided both *in cis* and *in trans*. AT-130 profoundly inhibited replication in both viral systems. Although this may imply no difference between encapsidation *in cis* or *in trans*, it is possible that a subtle difference in degree of inhibition was missed (von Weizsäcker et al., 1999, 2002).

As the only enzymatically active protein product of HBV, the polymerase protein has been the target of most HBV drug development. Because drug resistance has proven to be a significant problem, development of polymerase-independent inhibitors of HBV has become a priority. A number of small molecule inhibitors targeting HBV capsids have now been described. Zlotnick et al. (Zlotnick et al., 2002) showed that the novel molecule bis-ANS binds to the HBV capsid protein, inhibiting assembly of normal capsids and promoting assembly of non capsid polymers. Capsid protein bound to bis-ANS did not participate in assembly indicating a mechanism of inhibition analogous to competitive or non-competitive inhibition of enzymes. Deres and colleagues demonstrated that compounds of the heteroaryldihydropyrimidine (HAP) family, including BAY 41-4109, are able to inhibit HBV nucleocapsid formation and consequently HBV replication. HAPs were shown to bind core protein directly, leading to their degradation in the proteosome (Deres et al., 2003). The HAPs were also shown to cause an accelerated degradation of the unassembled capsid proteins. These compounds were active both *in vitro* and in a transgenic mouse model of hepatitis B (Weber et al., 2002). In contrast to these findings, AT-130 appears to inhibit encapsidation through an alternative mechanism since no reduction in nucleocapsid formation nor decrease in core protein levels was observed. This suggests that AT-130 does not interfere with the production of the components of encapsidation but rather with the molecular events involved in the process itself.

Although the development of nucleos(t)ide analogues as a class of inhibitors active against the HBV polymerase has been a major advancement in the treatment of chronic HBV infection, the emergence of resistance has remained a significant clinical problem. All of these compounds interfere with HBV DNA synthesis by their competitive incorporation in place of the natural nucleos(t)ide, resulting in chain termination of nascent HBV DNA. Unfortunately, single or multiple mutations in the polymerase gene are sufficient to confer high-level drug resistance to LMV (Allen et al., 1998) and ADV (Angus et al., 2003) and ETV(Colonno et al., 2006). More recent data has shown that the presence of resistance to one agent can predispose to the development of resistance to other agents in the class (Delaney et al., 2001). This problem is compounded by the fact that prolonged suppressive therapy is necessary to manage chronic hepatitis B, especially HBeAg-negative disease (Locarnini and Mason, 2006).

To deal with resistance effectively, it may become necessary, as has been shown with the success of highly active antiretroviral therapy (HAART), to target different stages in the viral lifecycle and develop non-nucleoside preferably, polymerase independent inhibitors of HBV replication. A few approaches have been evaluated. Glycosylation of the envelope proteins is necessary prior to virion export. Specific glucosidase inhibitors interfere with HBV replication. However, concerns have been raised about the potential toxicity of inhibiting such a widespread cellular process (Block et al., 1998). Various nucleic acid based therapies have been investigated including siRNA, ribozymes and

antisense RNA; however, to date technical difficulties such as hepatic delivery and *in vivo* stability have proven difficult to overcome (Loomba and Liang, 2006).

In conclusion, this study has demonstrated that the chemical class of compounds, the phenylpropenamides, can effectively and selectively inhibit HBV replication at a site independent of the reverse transcription process and probably at the level of pregenomic RNA encapsidation. Ultimately, development of agents targeting a variety of sites in the viral lifecycle will be very important to control in the long term ongoing HBV replication and combat the almost inevitable emergence of antiviral drug resistance to nucleos(t)ide analogues (Shaw and Locarnini, 2000).

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